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1) J Bacteriol 1967 May;93(5):1571-8
Glucose and gluconate metabolism in an Escherichia coli mutant lacking phosphoglucose isomerase.
Fraenkel DG, Levisohn SR.

2) Arch Microbiol 1989;151(5):466-8
Regulation of lysine decarboxylase activity in Escherichia coli K-12.
Auger EA, Bennett GN.

3) J Bacteriol 1982 Apr;150(1):52-9
Altered expression of biodegradative threonine dehydratase in Escherichia coli mutants.
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# Altered Expression of Biodegradative Threonine Dehydratase in *Escherichia coli* Mutants

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A number of strains of *Escherichia coli* K-12 failed to synthesize significant amounts of biodegradative threonine dehydratase (EC 4.2.1.16) when grown anaerobically in tryptone-yeast extract medium, a condition which is optimal for the induction of this enzyme. However, the addition of 10 mM potassium nitrate to the culture medium enabled a few of these strains, notably MB201, to induce the enzyme. An examination of the kinetic parameters, modifier sensitivity, and immunological cross-reactivity revealed that the enzyme produced by MB201 in nitrate-supplemented medium appeared indistinguishable from the dehydratase of a wild-type strain. The reduced expression of threonine dehydratase in MB201 appeared highly specific; the synthesis of two other inducible enzymes, D-serine deaminase and tryptophanase, and two "anaerobic" proteins, namely, fumarate reductase and cytochrome $c_{551}$, remained unaffected. The mutation (*tdcI*) responsible for the altered expression of the dehydratase in MB201 was located at min 91 on the *E. coli* chromosome and appeared to be tightly linked to if not identical with *pgi*, the gene encoding phosphoglucose isomerase, as judged by growth experiments on glucose and fructose, direct assay of phosphoglucose isomerase activity, spontaneous and simultaneous reversion of MB201 (TdcI) to TdcI$^+$ and Pgi$^+$ phenotype, and cosegregation of the two loci during transduction with P1 phage. Because not all strains lacking the dehydratase showed nitrate-dependent enzyme synthesis or had lesions at the *pgi* locus, it appears that mutations at multiple loci on the *E. coli* chromosome may influence the expression of the enzyme in vivo.

Although much is known regarding the structure and regulation of the purified biodegradative threonine dehydratases (EC 4.2.1.16) of *Escherichia coli* and *Salmonella typhimurium* (3, 5, 7, 18, 19, 23), the mechanism by which the synthesis of the enzyme is regulated in vivo is still unclear. Previous work has shown that the dehydratase is induced under anaerobic conditions in amino acid-rich medium, requires cAMP for its synthesis, and is sensitive to catabolite repression by glucose (20, 22, 26, 28). Recent studies (6, 29) on the amino acid requirements for enzyme synthesis indicate that a combination of several amino acids, especially threonine, valine, leucine, and aspartate, is essential for the phenomenon of multivalent induction. Because of the complex culture conditions required for the synthesis of the enzyme and lack of phenotype, it is difficult to select mutants affecting the enzyme, a factor which has delayed progress in a genetic analysis of this inducible system. By screening a number of strains of *E. coli* K-12, we have identified several which do not synthesize threonine dehydratase in the amino acid-rich tryptone-yeast extract medium. In this communication we present experimental evidence for a mutation that alters the inducibility of threonine dehydratase, its chromosomal location, and its physiological effects on enzyme synthesis.

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## MATERIALS AND METHODS

**Materials.** Tryptone, yeast extract, agar, and Freund complete adjuvant were supplied by Difco Laboratories. Amino acids were purchased from Sigma Chemical Co., 4-dimethylaminocinnamaldehyde was obtained through Aldrich Chemical Co., and Affigel-10 was purchased from Bio-Rad Laboratories. Purified biodegradative threonine dehydratase from *E. coli* K-12 was a generous gift from Linda Park (19).

**Bacteria and phage.** Table 1 lists strains of *E. coli*, their source, and relevant genotypes. Phage P1 *cml clr*100 was a gift from James J. Anderson of this department (1).

**Culture conditions.** Cells were routinely grown aerobically at 37°C in LB medium (17); the minimal medium used was medium A (17). To induce threonine dehydratase, cultures were incubated at 37°C in the tryptone-yeast extract medium (26); unless specified otherwise, strictly anaerobic conditions were maintained with a GasPak (BBL Microbiology Systems, Cockeysville, Md.), and the absence of oxygen was confirmed with a methylene blue indicator. In some

experiments, anaerobic conditions were approximated by filling the flasks or culture tubes to within 1 inch (ca. 2.5 cm) of the neck and allowing the vessels to remain stationary (still culture).

**Enzyme assays.** Threonine dehydratase activity in extracts was determined spectrophotometrically at 310 nm as described by Shizuta et al. (24). The assay mixture, in a final volume of 1 ml, contained 100 mM potassium phosphate buffer, pH 8.0, 50 mM L-threonine, 3 mM AMP, and 10 mM L-isoleucine (the latter to inhibit the activity of biosynthetic threonine dehydratase, if present).

The following procedure was routinely used to screen cultures for threonine dehydratase activity in whole cells. Fifty microliters of cell suspension, mixed with 5 μl of toluene, was shaken vigorously for 30 s. The toluenized cells were then incubated at 37°C for 20 min in 0.95 ml of the assay solution described above, and the amount of α-ketobutyrate produced was measured by the colorimetric assay method with dinitrophenylhydrazine (10) as modified by Bhadra and Datta (3).

Tryptophanase activity was determined by the cinnamaldehyde reagent according to Gartner and Riley (11), and D-serine deaminase activity was determined as described by McFall (16). Fumarate reductase activity was measured by monitoring fumarate-dependent oxidation of reduced benzyl viologen as described by Spencer and Guest (25) with the exception that benzyl viologen was photoreduced by using a proflavine catalyst (15); a unit of activity is defined as the amount of enzyme required to reduce 1 μmol of fumarate in 5 min at 0°C (benzyl viologen undergoes a spectral shift between 0 and 37°C). NADH oxidase activity was measured spectrophotometrically at 340 nm (13), and cytochrome $c_{551}$ content was determined from the difference spectra of oxidized minus dithionite-reduced samples (13). Phosphoglucose isomerase activity was measured by the glucose 6-phosphate coupled assay as recommended by Fraenkel and Levisohn (8). Enzyme activities are expressed as units of activity per milligram of protein; unless otherwise noted, a unit of enzyme is defined as the amount required to produce an increase in absorbance (at the appropriate wavelength) of 1.00 after incubation for 5 min at 37°C. Protein concentration was determined by the method of Lowry et al. (14).

**Preparation of antibodies.** A solution of purified threonine dehydratase (1 mg) emulsified with Freund complete adjuvant was administered to each of three rabbits by intradermal injections along the dorsal midline, and the procedure was repeated 120 days later. Ten days after the second injection, a small amount of blood was collected at weekly intervals, and antisera having a high titer of antibody were pooled. Antibodies were detected by the Ouchterlony double-diffusion technique (12). To purify antibodies, pooled antiserum was passed through a column of Affigel-10 to which purified threonine dehydratase had been coupled covalently (4). After exhaustive washing of the column with solutions of 0.15 M NaCl in 0.01 M sodium phosphate buffer, pH 7.2, and 1.4 M NaCl in the sodium phosphate buffer, antibodies were eluted with 4.5 M $MgCl_2$, essentially as described by Shapiro et al. (21).

**Selection of revertants.** Strain MB201 produces

TABLE 1. *E. coli* strains used

| Strain | Genotype | Source |
|---|---|---|
| T211B | F⁻ *ara-14 mtl-1 xyl-5 leu-6 proC34 trp-43 purE38 thi-1 lon azi-6 rpsL tonA23 tsx-67 tdcI* | A. Markovits via F. C. Neidhardt |
| KL14 | Hfr *thi-1 relA1* λ⁻ | J. J. Anderson |
| KL16 | Hfr *thi-1 relA1* λ⁻ | J. J. Anderson |
| KL226 | Hfr *relA1 tonA22* λ⁻ | J. J. Anderson |
| PK191 | Hfr Δ(*proB-lac*) *sup-56 x111* λ⁻ | J. J. Anderson |
| HfrH | Hfr *thi-1 rel-1* λ⁻ | J. J. Anderson |
| ES430 | Hfr *thi-1 malB29 relA1* λ⁻ | B. J. Bachmann |
| BW113 | Hfr *metB1* λ⁻ | J. J. Anderson |
| AB1927 | Hfr *metA28 argH1 purF1 xyl-7 supE44*? | B. J. Bachmann |
| DF40 | Hfr *pgi-2 relA1 tonA22* T2ʳ | B. J. Bachmann |
| M2508 | Hfr *metB1 relA1 melA7* | B. J. Bachmann |
| JP5053 | F⁻ *argH1 metB1 nagA1 rpsL155 rpoB352* | J. J. Anderson |
| CU372 | F⁻ *gal* Δ(ilvDAC) *leu-455* | S. C. Quay |
| AE84 | F⁻ *argG6 his-1 trp-31 mtl-2 xyl-7 malA1 gal-6 lacY1* or *lacZ4 rpsL tonA2 tsx-1* λʳ λ⁻ *supE44 thyA pdxC nalA* | J. J. Anderson |
| W4680 | F⁻ *lacZ39 metB4 rpsL45* or *rpsL-110* | |
| EO300 | F⁻ λ⁻ | B. J. Bachmann |
| MB102 | *rpoB*, otherwise as CU372 | J. J. Anderson |
| MB201[a] | F⁻ *proC34 purE38 rpsL trp-43 tdcI* | This study |
| MB202 | *tdcI*⁺, otherwise as MB201 | Conjugation, PK191 × T211B |
| MB203 | *argH1 metB1 rpoB352*, otherwise as MB201 | This study (see text) |
| MB431 | *rpoB*, otherwise as ES430 | *rpoB* transductant of MB201 from JP5053 |
| MB206 | *malB29 rpoB*, otherwise as MB201 | This study |
| MB227 | *malB29 rpoB*, otherwise as KL226 | *rpoB* transductant of MB201 from MB431<br>*rpoB* transductant of KL226 from MB431 |

[a] This strain may also carry additional parental markers.

TABLE 2. Specific activity of threonine dehydratase induced in selected strains of *E. coli* K-12[a]

| Strain | Culture density[b] | Sp act |
|---|---|---|
| AB1927 | 0.97 | <0.2 |
| M2508 | 0.50 | 0.4 |
| AE84 | 1.48 | 0.7 |
| ES430 | 0.56 | 1.3 |
| KL14 | 1.46 | 1.7 |
| W4680 | 1.46 | 2.0 |
| MB201 | 1.32 | 2.7 |
| HfrH | 1.62 | 22.1 |
| EO300 | 1.55 | 22.6 |
| DF40 | 0.97 | 22.9 |
| MB202 | 1.87 | 33.6 |
| BW113 | 1.46 | 60.9 |
| KL16 | 1.53 | 77.4 |
| MB227 | 1.19 | 91.4 |

[a] Ten milliliters of tryptone-yeast extract medium was inoculated with 0.1 ml of an aerobic overnight culture (initial absorbancy at 550 nm = 0.08) of the designated strain and cultivated anaerobically for 24 h. Enzyme activity was measured in toluene-treated cells as described in Materials and Methods.

[b] Absorbance at 550 nm.

greatly reduced levels of threonine dehydratase during anaerobic growth in tryptone-yeast extract medium (Table 2 and Fig. 1). Spontaneous revertants of MB201, which produces as much enzyme as most wild-type strains in tryptone-yeast extract medium, were isolated as follows. MB201 was cultured anaerobically in the tryptone-yeast extract medium. After 24 h (20 h after the cessation of exponential growth; see Fig. 1A), a gradual increase in culture density accompanied the appearance of dehydratase activity. From a sample taken at 54 h, several single colonies were isolated and purified. One of these clones, designated MB202, which showed the usual induction kinetics of the dehydratase in tryptone-yeast extract, was used for further experiments; this strain had the other auxotrophic markers (see Table 1) present in the parent MB201 as determined by plating on appropriate media. Although the process by which prolonged cultivation of MB201 in tryptone-yeast extract selects for revertants exhibiting enzyme induction is not understood, three separate experiments produced independent clones with threonine dehydratase activity.

**Conjugation and transduction studies.** Gradients of transmission in uninterrupted mating experiments were determined according to the method of Anderson et al. (1). Generalized transduction was performed, using the phage P1 *cml clr*100 in the manner described by Miller (17).

## RESULTS

**Variability in enzyme induction in various strains of *E. coli* K-12.** A number of strains of *E. coli*, obtained from several laboratories, exhibited various levels of threonine dehydratase activity when cultivated anaerobically in tryptone-yeast extract medium (Table 2). With two exceptions (M2508 and ES430), all strains having lower specific activities grew to similar culture densities compared with those with higher enzyme levels, indicating no obvious relationship between cell growth and enzyme activity.

**Cell growth and enzyme induction in strains MB201 and MB202.** To examine more carefully the physiological effect of a low level of threonine dehydratase, we measured the kinetics of enzyme induction and relative growth of strain MB201 and a spontaneous revertant of this strain, designated MB202 (see Materials and Methods). Throughout a 14-h growth period, MB201 had negligible enzyme activity, whereas MB202 showed maximal enzyme synthesis after about 9 h (Fig. 1C). A comparison of relative growth of MB201 and MB202 in tryptone-yeast extract medium showed that MB202 grew to twice the turbidity reached by MB201 (Fig. 1A). However, when the medium was supplemented with 10 mM $KNO_3$, an electron acceptor that generally improves cell growth in an anaerobic environment (27), both strains eventually reached the same culture density (Fig. 1B). Although the significance of this finding remains to be established, a more striking observation was that a threonine dehydratase activity was induced in MB201 growing anaerobically in the presence of nitrate (Fig. 1D). In several independent experiments, the maximal amount of threonine dehydratase found in nitrate-grown MB201 varied between 50 and 100% that seen in MB202 (cf. Fig. 2) or a wild-type K-12 strain; sodium nitrate was equally effective as potassium nitrate. The addition of two other physiologically

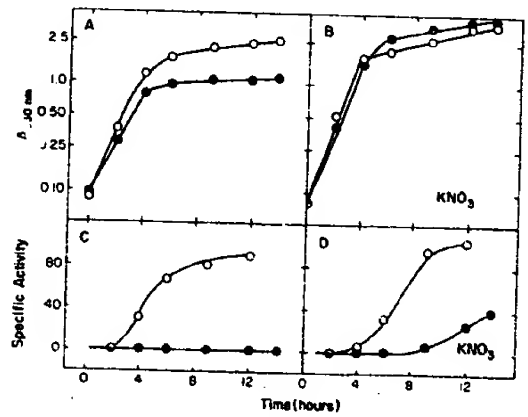


FIG. 1. Growth and induction of threonine dehydratase activities in strains MB201 (●) and MB202 (○). Cultures were grown in still culture in tryptone-yeast extract (A, C) or in the same medium supplemented with 10 mM $KNO_3$ (B, D). Cell growth is expressed as increase in absorbance at 550 nm measured after appropriate dilution. Enzyme activity was assayed as described in Materials and Methods and expressed as units per milligram of protein.

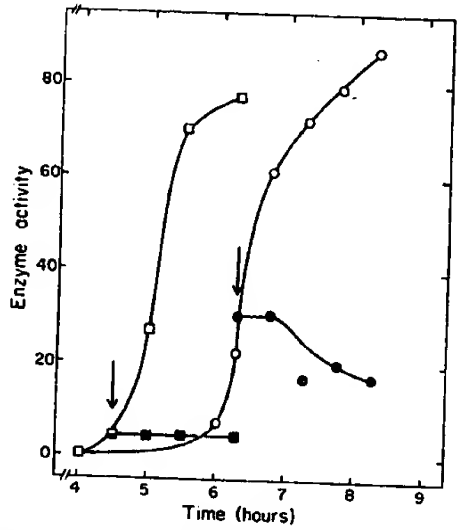


FIG. 2. Effect of chloramphenicol on the induction of threonine dehydratase in strains MB201 (○, ●) and MB202 (□, ■). Cultures were grown in still culture in the tryptone-yeast extract medium supplemented with 10 mM $KNO_3$. At various times, enzyme activity was assayed as described in the Materials and Methods and expressed as units per milligram of protein. To separate cultures (■, ●) chloramphenicol (100 μg/ml) was added at times indicated by the arrows. No chloramphenicol was added in control cultures (□, ○).

functional electron acceptors, such as fumarate (40 mM) and nitrite (5 mM), to the cultures of MB201 also resulted in the induction of threonine dehydratase (data not shown).

It is possible to argue that MB201 produces an altered form of the dehydratase which is unstable in cells grown in tryptone-yeast extract medium but is stabilized in the presence of electron acceptors. This notion was ruled out by the following experiments. Dialysis of an extract from nitrate-grown MB201 against 0.1 M potassium phosphate buffer, pH 8.0, in the presence or absence of 10 mM $KNO_3$ (with or without 3 mM AMP) did not affect the specific activity of the enzyme; furthermore, addition of nitrate during assay to extract free of nitrate did not increase enzyme activity. Similar results were seen with extracts from MB202 grown in the presence of nitrate.

Addition of chloramphenicol to cultures of MB201 and MB202 soon after the onset of enzyme induction prevented further increase in the level of the enzyme (Fig. 2), indicating that cultivation of MB201 in the nitrate-supplemented tryptone-yeast extract medium resulted in de novo synthesis of threonine dehydratase.

**Characterization of the enzyme from MB201.** The ability of the extracts from MB201 grown in tryptone-yeast extract with nitrate to catalyze the threonine dehydration reaction prompted us to examine whether the catalytic activity observed was due to the presence of biodegradative threonine dehydratase, or whether the extract contained a new enzyme activity elaborated during growth in nitrate-supplemented medium. Several lines of evidence described below clearly showed that the dehydratase of MB201 was similar to the enzyme isolated from the revertant MB202 or from the wild-type *E. coli*.

It is well established (5, 19, 23) that the activity of biodegradative threonine dehydratase is stimulated by AMP and that the enzyme can be purified by affinity chromatography on AMP-Sepharose. Furthermore, the enzyme is subject to catabolite inactivation by several intermediary metabolites, notably pyruvate and glyoxylate (7, 19). We found that the dehydratase isolated from nitrate-grown MB201 was stimulated by AMP, and the AMP-free enzyme bound to AMP-Sepharose; it was eluted by AMP to achieve a high degree of purity. The $K_m$ values for L-threonine in the presence of a saturating concentration of AMP were 5.1 mM and 3.7 mM, respectively, for enzymes isolated from MB201 and MB202.

As with MB202, incubation of extracts from MB201 with pyruvate or glyoxylate resulted in a time-dependent loss of enzyme activity. A comparison of the inactivation kinetics showed that, in 60 min, 20 mM glyoxylate inactivated 25% of

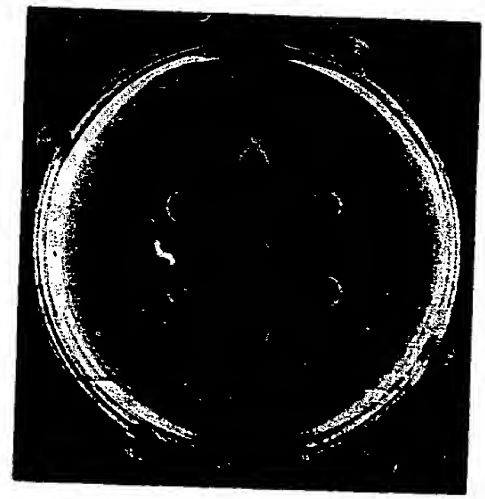

FIG. 3. Immunological analysis of enzymes in extracts of strains MB201 and MB202. The center well contained 10 μg of affinity-purified antibodies (rabbit) directed against purified threonine dehydratase from *E. coli* K-12. Wells 1 and 2, Extracts of MB202 (500 μg) and MB201 (460 μg), respectively, from cells grown in tryptone-yeast extract medium; well 3, extract of MB201 (560 μg) grown in $KNO_3$-supplemented tryptone-yeast extract medium; wells 4 and 6, 10 μg of purified *E. coli* K-12 enzyme; well 5, buffer.

TABLE 3. Levels of selected catabolic enzymes in strains MB201 and MB202 grown in the presence or absence of $KNO_3$[a]

| Enzyme | Sp act | | | |
|---|---|---|---|---|
| | MB201 | | MB202 | |
| | $-KNO_3$ | $+KNO_3$ | $-KNO_3$ | $+KNO_3$ |
| Threonine dehydratase | 0.77 | 32.7 | 36.7 | 43.4 |
| Tryptophanase | 0.15 | 0.26 | 0.29 | 0.30 |
| D-Serine deaminase | 31.5 | 20.7 | 38.1 | 22.8 |

[a] Cells were grown in still culture at 37°C in tryptone-yeast extract medium (for threonine dehydratase) or in the same medium supplemented with 1 mg of L-tryptophan per ml (for tryptophanase) or 0.5 mg of D-serine per ml (for D-serine deaminase). Where indicated, the medium also contained 10 mM $KNO_3$. Enzyme activities of toluenized cells were assayed as described in Materials and Methods.

the dehydratase from MB201 and about 15% of activity from MB202; with 20 mM pyruvate, the rates of enzyme inactivation were identical with both extracts. These results are reminiscent of those seen with the wild-type enzyme, and the small difference in the rates of inactivation by glyoxylate may be attributable to the difference in the protein concentrations in the incubation mixture (cf. references 7, 19).

Further evidence for the identity of the enzymes was revealed by the Ouchterlony double-diffusion immunoprecipitation technique (Fig. 3). An extract of MB201 grown in nitrate-supplemented medium showed a precipitin band with affinity-purified antibodies raised against purified threonine dehydratase from *E. coli* K-12; the line of identity (without a spur) between the wild-type and MB201 enzymes indicates that they were immunologically identical. By the same evidence, the enzyme in the extract of the revertant MB202 was also identical to the wild-type enzyme. It is noteworthy that there was no cross-reacting material in the extract of MB201 grown in the tryptone-yeast extract medium without $KNO_3$.

**Synthesis of other inducible enzymes in MB201.** Biodegradative threonine dehydratase is one of several enzymes that requires cAMP for its synthesis and is subject to catabolite repression by glucose (20, 22). Conceivably, the mutation in MB201 may alter the requirements for cAMP for the induction of threonine dehydratase. However, the addition of 10 mM cAMP, which allows threonine dehydratase synthesis in adenyl cyclase mutants (20), did not result in enzyme production in MB201. The defect in MB201 also did not affect the induction of D-serine deaminase (EC 4.2.1.14) and tryptophanase (EC 4.1.99.1) in the tryptone-yeast extract medium with and without $KNO_3$ and supplemented with the specific inducers for these enzymes. Whereas threonine dehydratase levels could vary over 40-fold, there was little difference in the levels of D-serine deaminase or tryptophanase between MB201 and the revertant (Table 3). More important, addition of nitrate did not significantly alter the induced levels of these enzymes in either MB201 or MB202. These results appear to suggest that the mutation in MB201 affects the expression of threonine dehydratase by a mechanism not common to the other two inducible enzymes.

**Synthesis of anaerobic proteins by strain MB201.** To determine whether strain MB201 is generally defective in adapting to anaerobic conditions, two proteins, fumarate reductase and cytochrome $c_{551}$, specifically involved in anaerobic metabolism, were measured in extracts of strains MB201, MB202, and MB227 cultivated anaerobically in tryptone-yeast extract medium. There was little variation in the levels of these proteins among the three strains (Table 4); in contrast, high levels of threonine dehydratase were found in MB202 and MB227 with negligible activity in MB201. In all cases, the rates of succinate oxidation by extracts (data not shown) were significantly lower than the rates of fumarate reduction, indicating that the enzyme responsible for fumarate reduction was most likely fumarate reductase and not succinate dehydrogenase (25). NADH oxidase activity, which is usually low during anaerobic growth (13), also did not vary significantly among strains MB201, MB202, and MB227 cultivated anaerobically (Table 4); as expected, the enzyme activity was increased approximately 15-fold in extracts of all three strains cultivated aerobically (data not shown).

**Chromosomal location of the mutation in MB201.** The cumulative results described above clearly suggest that a mutation in MB201 (tentatively designated *tdcI*) somehow prevents the

TABLE 4. Levels of several proteins involved in aerobic or anaerobic metabolism in strains MB201, MB202, and MB227 cultivated anaerobically[a]

| Strain | Protein content | | | |
|---|---|---|---|---|
| | Fumarate reductase | Cytochrome $c_{551}$ | Threonine dehydratase | NADH oxidase |
| MB201 | 280 | $3.8 \times 10^{-4}$ | 0.01 | 0.515 |
| MB202 | 319 | $2.6 \times 10^{-4}$ | 42.8 | 0.425 |
| MB227 | 213 | $3.7 \times 10^{-4}$ | 134.7 | 0.225 |

[a] Cultures were grown anaerobically for 12 h. Enzyme activities were determined in sonic extracts; cytochrome $c_{551}$ content was measured in the soluble fraction (13). All values are expressed as units per milligram of protein.

TABLE 5. Cotransduction frequencies of *tdcI* with known genetic loci

| Expt | Donor | Recipient | Selected marker | % Cotransduction with *tdcI* allele[a] |
|---|---|---|---|---|
| 1 | MB102 (*rpoB tdcI*$^+$)[b] | MB201 (*rpoB*$^+$ *tdcI*) | *rpoB* | 1.5 (3/200) |
| 2 | MB102 (*rpoB tdcI*$^+$) | MB201 (*rpoB*$^+$ *tdcI*) | *rpoB* | 1 (1/100) |
| 3 | MB102 (*metB*$^+$ *tdcI*$^+$) | MB203 (*metB tdcI*) | *metB*$^+$ | 0 (0/50) |
| 4 | MB102 (*malB*$^+$ *tdcI*$^+$) | MB206 (*malB tdcI*) | *malB*$^+$ | 59.1 (117/198) |
| 5 | MB202 (*malB*$^+$ *tdcI*$^+$) | MB206 (*malB tdcI*) | *malB*$^+$ | 91 (91/100) |

[a] Unselected marker.
[b] Relevant genotype is given in parentheses.

induction of threonine dehydratase in tryptone-yeast extract medium; addition of an electron acceptor to the culture medium allows restoration of enzyme synthesis. To locate the mutation on the *E. coli* chromosome, three uninterrupted mating experiments were performed, using strain T211B as the recipient; as shown in Table 1, this strain contains several auxotrophic markers as well as a lesion in *tdcI* (as evidenced by the lack of threonine dehydratase induction in the absence of nitrate). For each cross, a proximal marker was selected on appropriate medium, and recombinants were scored for receipt of two additional known markers; the activity of threonine dehydratase in the recombinants was monitored by direct enzyme assay. From the gradients of transmission of the known markers (shown in Fig. 4), the mutation which results in the loss of enzyme synthesis in T211B appeared to lie between 87 and 92 min.

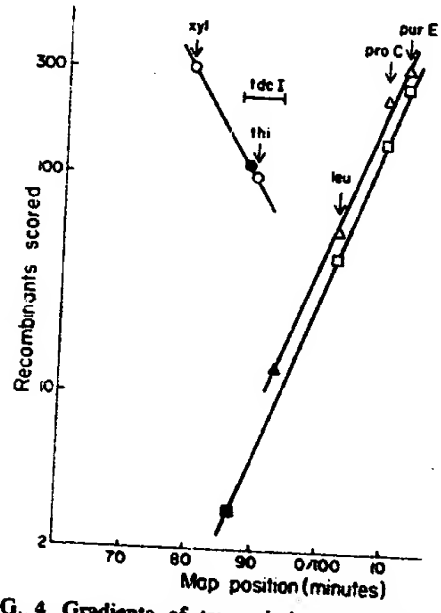


FIG. 4. Gradients of transmission of unselected markers in matings of the Hfr strains PK191 (○), KL16 (△), and KL226 (□) (all *tdcI*$^+$) with the *tdcI* recipient strain, T211B. Proximal markers selected were *xyl* for PK191 and *purE* for KL16 and KL226. Unselected markers were *thi* for PK191 and *proC* and *leu* for KL16 and KL226. Closed symbols are for *tdcI*.

To more precisely locate the mutation within this region, two-factor crosses by transduction with P1 phage were performed (Table 5). For these experiments, MB201 (obtained as a recombinant from a cross between PK191 × T211B; see Table 1) was used as a *tdcI* recipient. Experiments 1 through 3 illustrate that *tdcI* was not linked to *metB* (86 min) but was weakly linked to *rpoB* (90 min) with a cotransduction frequency of less than 2%. The results of experiment 4 show that *tdcI* was cotransduced with the *malB* region (91 min) at a high frequency. The results of experiment 5 indicate that the spontaneous mutation which yielded MB202 from MB201 was also strongly linked to *malB*.

**Identity of the *tdcI* and *pgi* loci.** After the chromosomal location of *tdcI* became known, this region of the *E. coli* genetic map (2) was examined for any known genes that might confer the phenotype in MB201. Fraenkel and his collaborators (8, 9) have observed that the *pgi*

TABLE 6. Anaerobic growth of strains MB201, MB202, and DF40 on glucose and fructose[a]

| Strain | Turbidity[b] | |
|---|---|---|
| | Glucose | Fructose |
| MB201 (*tdcI*) | 0 | 90 |
| MB202 (*tdcI*$^+$) | 98 | 70 |
| DF40 (*pgi*) | 0 | 83 |
| MB227 (*tdcI*$^+$ *pgi*$^+$) | 77 | 57 |

[a] Ten milliliters of minimal medium was inoculated with $10^7$ washed cells of the appropriate strain. Cultures were incubated anaerobically for 24 h.
[b] Determined with a Klett-Summerson photoelectric colorimeter, using a red filter.

TABLE 7. Phosphoglucose isomerase activity in extracts of several strains of *E. coli* K-12[a]

| Strain | Protein (μg) | Sp act |
|---|---|---|
| MB201 | 16.5 | 0 |
| | 330 | 0 |
| MB202 | 16.0 | 86.5 |
| MB227 | 16.0 | 54.5 |
| DF40 | 14.0 | 0 |
| | 280 | 0 |
| MB202 | 8.0 | 102.0 |
| MB202 + MB201 | 8.0<br>8.0 | 100.5[b] |
| MB202 + MB201 | 8.0<br>16.0 | 106.7[b] |

[a] Extracts were prepared from cells cultivated anaerobically in tryptone-yeast extract medium.
[b] Expressed as milligrams of protein from MB202 extract.

locus, which encodes phosphoglucose isomerase, is closely linked to *malB*; mutants lacking this enzyme grow slowly on glucose aerobically (8), but not at all anaerobically (9), and can utilize fructose efficiently under either condition. MB201 exhibited the same growth characteristics as strain DF40, a known *pgi* mutant (9) (Table 6). The spontaneous revertant, MB202, grew well on either glucose or fructose anaerobically as did the wild-type strain, MB227.

The direct evidence for the identity of the *tdcI* locus with that of *pgi* was obtained from assay of phosphoglucose isomerase activities in MB201 and DF40 (Table 7): extracts of these strains contained no isomerase activity, whereas extracts of the spontaneous *tdcI*$^+$ revertant (MB202) and a wild-type strain (MB227) contained similar amounts of enzyme. Control experiments with mixed extracts from the mutant and revertant showed no evidence for an inhibitor of phosphoglucose isomerase in the extract of MB201 (Table 7).

In a genetic experiment, a *malB* derivative of MB201 was transduced to *malB*$^+$, using phage P1 grown on MB202, and *tdcI* and *pgi* were found to cosegregate in 50/50 transductants. These data, taken together, strongly suggest that *tdcI* is tightly linked if not identical to *pgi*.

**Effect of nitrate on enzyme synthesis in various *E. coli* K-12 strains.** Seven of 14 *E. coli* K-12 strains listed in Table 2 had low levels of threonine dehydratase when grown anaerobically in tryptone-yeast extract medium. One of these strains, MB201, showed 50- to 100-fold more enzyme when nitrate was added to the culture medium. To examine whether nitrate also enhanced enzyme synthesis in other strains with low activity, cultures were grown anaerobically in tryptone-yeast extract medium with or without nitrate, and enzyme activities were determined in cell extracts. Strains AE84 and DF40 produced significantly more enzyme in the presence of nitrate (Table 8); in the other strains, nitrate did not enhance enzyme synthesis, suggesting that a separate mechanism might be responsible for the low level of enzyme synthesis. (An intermediate level of threonine dehydratase synthesis in DF40 in tryptone-yeast extract medium and stimulation of enzyme induction by nitrate suggested that DF40 is partially affected by the *pgi* lesion in this strain.)

## DISCUSSION

Despite some recent progress in defining culture conditions which influence the synthesis of the biodegradative threonine dehydratase, the true nature of the regulatory molecule(s) still remains elusive. Several amino acids, notably threonine, valine, leucine, and aspartate, appear necessary for maximal enzyme synthesis (6, 29), and the requirement for an anaerobic environment is presumably linked to intracellular levels of cyclic AMP (20). We report here that a mutation at 91 min on the *E. coli* chromosome, which is most likely a lesion at the *pgi* locus encoding phosphoglucose isomerase, prevents enzyme induction unless a physiologically functional electron acceptor such as nitrate is present in the culture medium. Although the relationship between *pgi*, threonine dehydratase, and nitrate are unclear, it is conceivable that an internally generated metabolite (acting either as an inducer or a repressor) may regulate enzyme synthesis under anaerobic conditions; in a *pgi* mutant, a block in gluconeogenesis could alter the concentration of this putative regulatory molecule, whereas addition of an electron acceptor would open new metabolic pathways (27) restoring normal levels of this metabolite to allow enzyme synthesis. The role of a cellular "metab-

TABLE 8. Effect of $KNO_3$ on induction of threonine dehydratase in selected *E. coli* strains[a]

| Strain | Sp act | |
|---|---|---|
| | $-KNO_3$ | $+KNO_3$ |
| MB201 | 0.32 | 15.73 |
| AE84 | 0.37 | 5.94 |
| DF40 | 8.93 | 32.62 |
| M2508 | <0.20 | <0.20 |
| AB1927 | <0.20 | <0.20 |
| ES430 | 1.05 | 0.89 |
| KL14 | 4.73 | 4.00 |
| W4680 | 8.17 | 6.83 |

[a] Ten milliliters of tryptone-yeast extract medium with or without 10 mM $KNO_3$ was inoculated with an aerobic overnight culture (0.1 ml) of the designated strain. Threonine dehydratase activity of toluene-treated cells was determined after 24 h of anaerobic cultivation.

olite" in enzyme synthesis has also been inferred by Yui et al. (29) to explain the phenomenon of multivalent induction; Phillips et al. have also suggested (20) that pyruvate may repress the formation of threonine dehydratase. It is noteworthy in this context that threonine dehydratase levels in a particular strain vary somewhat, possibly because of parameters difficult to control in a complex medium, and that strains of *E. coli* having little or no dehydratase activity are, surprisingly, of common occurrence (cf. Table 2), and not all these strains show enhanced enzyme induction in the presence of nitrate (cf. Table 8). Furthermore, preliminary mapping data (D. Merberg, Ph.D. thesis, The University of Michigan, 1981) with KL14 and ES430 revealed that the threonine dehydratase-negative phenotype is not linked to lesions at the *pgi* locus. These observations are consistent with the notion that a complex regulatory system controls the synthesis of this enzyme, and mutations at different loci affecting the intracellular concentrations of a variety of metabolites may influence enzyme induction. Continuing studies on the nitrate-dependent enzyme production by *pgi* mutants in various defined media, and analyses of other regulatory loci involved in enzyme synthesis, should provide a better insight into the mechanism of induction of the biodegradative threonine dehydratase in the coliaerogenes group of bacteria.

## ACKNOWLEDGMENTS

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## LITERATURE CITED

1. Anderson, J. J., S. C. Quay, and D. L. Oxender. 1976. Mapping of two loci affecting the regulation of branched chain amino acid transport in *Escherichia coli* K-12. J. Bacteriol. 126:80–90.
2. Bachmann, B. J., and K. B. Low. 1980. Linkage map of *Escherichia coli* K-12, edition 6. Microbiol. Rev. 44:1–56.
3. Bhadra, R., and P. Datta. 1978. Allosteric inhibiton and catabolite inactivation of purified biodegradative threonine dehydratase of *Salmonella typhimurium*. Biochemistry 17:1691–1699.
4. Cuatrecasas, P., and I. Parikh. 1972. Adsorbents for affinity chromatography. Use of N-hydroxysuccinimide esters of agarose. Biochemistry. 11:2291–2299.
5. Dunne, C. P., and W. A. Wood. 1975. L-Threonine dehydrase as a model of allosteric control involving ligand-induced oligomerization. Curr. Top. Cell. Regul. 9:65–101.
6. Egan, R. M., and A. T. Phillips. 1977. Requirements for induction of biodegradative threonine dehydratase in *Escherichia coli*. J. Bacteriol. 132:370–376.
7. Feldman, D. A., and P. Datta. 1975. Catabolite inactivation of biodegradative threonine dehydratase of *Escherichia coli*. Biochemistry 14:1760–1767.
8. Fraenkel, D. G., and S. R. Levisohn. 1967. Glucose and gluconate metabolism in an *Escherichia coli* mutant lacking phosphoglucose isomerase. J. Bacteriol. 93:1571–1578.
9. Fraenkel, D. G., and R. T. Vinopal. 1973. Carbohydrate metabolism in bacteria. Annu. Rev. Microbiol. 27:69–100.
10. Friedemann, T. E., and G. E. Haugen. 1943. Pyruvic acid. II. The determination of keto acids in blood and urine. J. Biol. Chem. 147:415–431.
11. Gartner, T. K., and M. Riley. 1965. Isolation of mutants affecting tryptophanase production in *Escherichia coli*. J. Bacteriol. 89:313–318.
12. Goldstein, I. J., and L. L. So. 1965. Protein-carbohydrate interaction. III. Agar gel-diffusion studies on the interaction of Concanavalin A, a lectin isolated from jack bean, with polysaccharides. Arch. Biochem. Biophys. 111:407–414.
13. Gray, C. T., J. W. T. Wimpenny, D. E. Hughes, and M. R. Mossman. 1966. Regulation of metabolism in facultative bacteria. I. Structural and functional changes in *Escherichia coli* associated with shifts between the aerobic and anaerobic states. Biochim. Biophys. Acta 117:22–32.
14. Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. J. Randall. 1951. Protein measurement with the Folin phenol reagent. J. Biol. Chem. 193:265–275.
15. Massey, V., M. Stankovich, and P. Hemmerich. 1978. Photoreduction of flavoproteins and other biological compounds catalyzed by deazaflavins. Biochemistry 17:1–8.
16. McFall, E. 1975. *Escherichia coli* K12 mutant forming a temperature-sensitive D-serine deaminase. J. Bacteriol. 121:1074–1077.
17. Miller, J. H. 1972. Experiments in molecular genetics. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
18. Park, L. S., and P. Datta. 1979. Inhibition of *Escherichia coli* biodegradative threonine dehydratase by pyruvate. J. Bacteriol. 138:1026–1028.
19. Park, L. S., and P. Datta. 1979. The role of glyoxylate in the regulation of biodegradative threonine dehydratase of *Escherichia coli*. J. Biol. Chem. 254:7927–7933.
20. Phillips, A. T., R. M. Egan, and B. Lewis. 1978. Control of biodegradative threonine dehydratase inducibility by cyclic AMP in energy-restricted *Escherichia coli*. J. Bacteriol. 135:828–840.
21. Shapiro, D. J., J. M. Taylor, G. S. McKnight, R. Palacios, C. Gonzalez, M. L. Kiely, and R. T. Schimke. 1974. Isolation of hen oviduct ovalbumin and rat liver albumin polysomes by indirect immunoprecipitation. J. Biol. Chem. 249:3665–3671.
22. Shizuta, Y., and O. Hayaishi. 1970. Regulation of biodegradative threonine deaminase synthesis in *Escherichia coli* by cyclic adenosine 3',5'-monophosphate. J. Biol. Chem. 245:5416–5423.
23. Shizuta, Y., and O. Hayaishi. 1976. Regulation of biodegradative threonine deaminase. Curr. Top. Cell. Regul. 11:99–146.
24. Shizuta, Y., A. Kurosawa, K. Inoue, T. Tanabe, and O. Hayaishi. 1973. Regulation of biodegradative threonine deaminase. I. Allosteric inhibition of the enzyme by a reaction product and its reversal by adenosine 5'-monophosphate. J. Biol. Chem. 248:512–520.
25. Spencer, M. E., and J. R. Guest. 1973. Isolation and properties of fumarate reductase mutants of *Escherichia coli*. J. Bacteriol. 114:563–570.
26. Umbarger, H. E., and B. Brown. 1957. Threonine deamination in *Escherichia coli*. II. Evidence for two L-threonine deaminases. J. Bacteriol. 73:105–112.
27. Wimpenny, J. W. T., and J. A. Cole. 1967. The regulation of metabolism in facultative bacteria. III. The effect of nitrate. Biochim. Biophys. Acta 148:233–242.
28. Wood, W. A., and I. C. Gunsalus. 1949. Serine and threonine deaminases of *Escherichia coli*: activators for a cell free enzyme. J. Biol. Chem. 181:171–182.
29. Yui, Y., Y. Watanabe, S. Ito, Y. Shizuta, and O. Hayaishi. 1977. Multivalent induction of biodegradative threonine deaminase. J. Bacteriol. 132:363–369.

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1) J Bacteriol 1967 May;93(5):1571-8
Glucose and gluconate metabolism in an Escherichia coli mutant lacking phosphoglucose isomerase.
Fraenkel DG, Levisohn SR.

2) Arch Microbiol 1989;151(5):466-8
Regulation of lysine decarboxylase activity in Escherichia coli K-12.
Auger EA, Bennett GN.

3) J Bacteriol 1982 Apr;150(1):52-9
Altered expression of biodegradative threonine dehydratase in Escherichia coli mutants.
Merberg D, Datta P.

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# Glucose and Gluconate Metabolism in an *Escherichia coli* Mutant Lacking Phosphoglucose Isomerase

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A single gene mutant lacking phosphoglucose isomerase (*pgi*) was selected after ethyl methane sulfonate mutagenesis of *Escherichia coli* strain K-10. Enzyme assays revealed no *pgi* activity in the mutant, whereas levels of glucokinase, glucose-6-phosphate dehydrogenase, and gluconate-6-phosphate dehydrogenase were similar in parent and mutant. The amount of glucose released by acid hydrolysis of the mutant cells after growth on gluconate was less than 2% that released from parent cells; when grown in the presence of glucose, mutant and parent cells contained the same amount of glucose residues. The mutant grew on glucose one-third as fast as the parent; it also grew much slower than the parent on galactose, maltose, and lactose. On fructose, gluconate, and other carbon sources, growth was almost normal. In both parent and mutant, gluconokinase and gluconate-6-phosphate dehydrase were present during growth on gluconate but not during growth on glucose. Assay and degradation of alanine from protein hydrolysates after growth on glucose-*1*-$^{14}C$ and gluconate-*1*-$^{14}C$ showed that in the parent strain glucose was metabolized by the glycolytic path and the hexose monophosphate shunt. Gluconate was metabolized by the Entner-Doudoroff path and the hexose monophosphate shunt. The mutant used glucose chiefly by the shunt, but may also have used the Entner-Doudoroff path to a limited extent.

The properties of a mutant of *Salmonella typhimurium* deficient in a central enzyme of glycolysis, phosphoglucose isomerase, have been described previously (8,9). This mutant grew on glucose about one-fifth as fast as the parent strain, apparently largely by using glucose via the hexose monophosphate shunt. Enzymatic and isotopic data suggested that both parent and mutant strains used the shunt at about the same rate; thus, this pathway appeared to be nonexpandable and, indeed, to limit the growth rate of the mutant. Nevertheless, both parent and mutant grew at similar, high rates on gluconate, although this compound had been thought to be metabolized only via the shunt. However, isotopic experiments and measurement of enzymes (8) showed that gluconate was metabolized in *S. typhimurium* largely via the Entner-Doudoroff path (6); gluconokinase and the first enzyme of the Entner-Doudoroff path, gluconate-6-phosphate dehydrase were found to be induced by gluconate but not by glucose. Thus, when gluconate-6-phosphate arose from glucose its metabolism appeared to be restricted to the shunt, whereas when it arose from gluconate it was able to use the Entner-Doudoroff pathway (Fig. 1 shows the relevant pathways).

We have now also selected mutants of *Escherichia coli* lacking phosphoglucose isomerase. In this paper, we describe the properties of one such mutant. We have paid particular attention to the role of the Entner-Doudoroff path in gluconate and in glucose metabolism, partly because Loomis and Magasanik (12). in experiments with *E. coli* K-12 and a mutant lacking phosphoglucose isomerase derived from it (10), obtained results suggesting that the Entner-Doudoroff path may have a major role in glucose, as well as in gluconate metabolism. Our results differ from theirs in certain respects, and show that the pathways of glucose and gluconate metabolism in *E. coli* are similar to those of *S. typhimurium*.

## MATERIALS AND METHODS

*Chemicals.* Gluconate-*1*-$^{14}C$ was from Nuclear-Chicago Corp., Des Plaines, Ill. Glucose-*1*-$^{14}C$, L-alanine-*U*-$^{14}C$ and DL-alanine-*1*-$^{14}C$ were from New England Nuclear Corp., Boston, Mass. 2-Keto-3-deoxygluconate-6-phosphate was a generous gift from W. A. Wood. Sodium gluconate was from Eastman Organic Chemicals, Rochester, N.Y., and contained

<0.05% glucose. Glucose-6-phosphate dehydrogenase and lactic dehydrogenase were from Boehringer Mannheim Corp., New York, N.Y.; other biochemicals were from Boehringer or from Sigma Chemical Co., St. Louis, Mo.

*Assays.* Glucose was measured with glucose oxidase (Glucostat, Worthington Biochemical Corp., Freehold, N.J.). Protein was measured by the Folin method (14) with bovine plasma albumin as a standard, corrected for moisture content (11).

*Enzyme assays.* Cell-free extracts were prepared from 250- or 500-ml cultures. The cells were collected by centrifugation, washed once with 0.9% NaCl, and resuspended in 1% of the original volume of buffer containing 0.01 M tris(hydroxymethyl)aminomethane (Tris) chloride, 0.01 M $MgCl_2$, and 0.001 M dithiothreitol (*p*H 7.8). These suspensions were treated for 1 min per ml with an MSE Ultrasonicator, and then were centrifuged at 17,000 × *g* (maximum) for 30 min; the pellets were discarded. Incubation mixtures for all assays contained 0.05 M Tris chloride and 0.01 M $MgCl_2$ (*p*H 7.6). The nicotinamide adenine dinucleotide phosphate (NADP)-linked direct spectrophotometric assays were done with 1-ml final volumes, and contained 0.2 mM NADP. The other additions were as follows: for glucokinase, 0.5 mM glucose, 2mM adenosine triphosphate (ATP), and 1 μg of glucose-6-phosphate dehydrogenase (Boehringer Mannheim Corp.); for phosphoglucose isomerase, 0.4 mM fructose-6-phosphate and 1 μg of glucose-6-phosphate dehydrogenase; for glucose-6-phosphate dehydrogenase, 0.6 mM glucose-6-phosphate; for gluconate-6-phosphate dehydrogenase, 0.4 mM gluconate-6-phosphate; and for gluconokinase, 0.5 mM sodium gluconate and 2 mM ATP. 2-Keto-3-deoxygluconate-6-phosphate (KDGP) aldolase was measured as substrate-dependent reduced nicotinamide adenine dinucleotide ($NADH_2$) oxidation in a mixture with a total volume of 0.2 ml containing the usual buffer, 0.15 mM KDGP (barium salt), 0.1 mM $NADH_2$, and 1 μg of lactic dehydrogenase. In all these assays, the reactions were started by the addition of extract, and the change in absorption at 340 mμ was followed in a Gilford Model 2000 recording spectrophotometer with the cell chamber kept at 25 C. With one exception, the reactions were proportional to the amount of extract and were linear with time for several minutes. The exception was gluconate-6-phosphate dehydrogenase, whose rate fell as much as 40% in the first 2 min, possibly because of inactivation; for this enzyme, initial rates are given.

Gluconate-6-phosphate dehydrase was measured at room temperature in a two-step assay, as previously described (8). This assay depends on the excess of KDGP aldolase present in the extract.

*Organisms and media.* The HFr strain of *E. coli* K-12, called K-10, was used as the parent strain. Minimal medium 63 (8) was supplemented with thiamine hydrochloride (1 μg/ml) and the carbon source (4 mg/ml). The broth medium was 63 supplemented with 1% tryptone (Difco) and 0.4% glucose. Solid media contained 2% agar. For glucose tetrazolium indicator plates (16), 25.5 g of Antibiotic Medium No. 2 (Difco) and 50 mg of 2,3,5-triphenyl

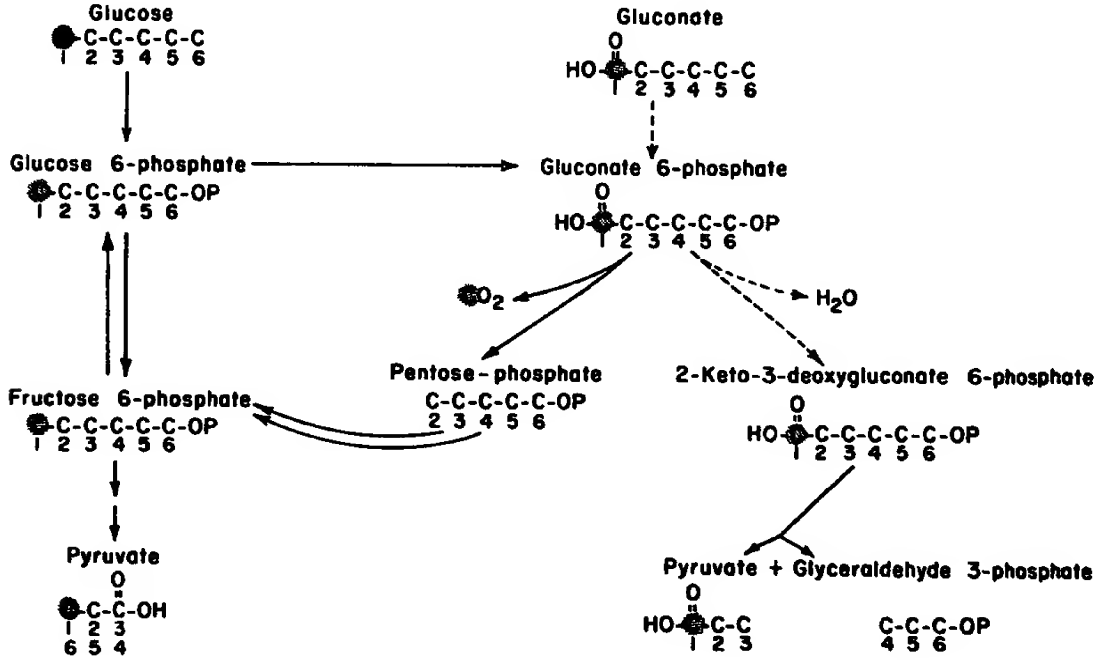


FIG. 1. *Pathways of glucose and gluconate metabolism. This figure outlines the major pathways of glucose and gluconate metabolism in Escherichia coli. Dashed arrows represent reactions known to be catalyzed by inducible enzymes. The distribution of the carbon atoms of glucose-1-$^{14}C$ and gluconate-1-$^{14}C$ are also sketched. The numbering is of the carbon atoms in the parent compounds.*

tetrazolium chloride (Sigma Chemical Co.) were dissolved in 950 ml of water and autoclaved for 30 min at 120 C; 50 ml of 20% glucose was added. Cultures were always incubated at 37 C.

*Growth rates.* Flasks (125-ml) fitted with side arms 14 mm in diameter and baffles on the bottom to increase aeration were used with 10 ml of culture, shaking on a New Brunswick Gyrotory water bath shaker at 37 C, at 220 cycles per min of 1.1-cm excursion. Turbidity was followed on a Lumetron colorimeter model 401 with a 580 filter; the readings were corrected for their deviation from proportionality to bacterial concentration and were translated to bacterial dry weight on the basis of a dry-weight determination of a measured quantity of K-10 in logarithmic growth on glucose. The turbidity range in growth rate experiments was 0.025 to 0.75 mg (dry weight) per ml. Growth rates were determined from the slopes of plots of bacterial dry weight (on a logarithmic scale) versus time.

*Glucose utilization.* Cultures were grown in a medium containing, initially, 2 mg of glucose per ml. Samples (0.5 ml) were immediately frozen and were later thawed and centrifuged in the cold to remove cells. Glucose was assayed on portions of the supernatant fluid, and the values obtained were plotted against dry weight as determined from the turbidity measurements.

*Glucose content of hydrolysates.* Strains K-10 and L40 were inoculated in dilutions of 1:500 and grown to stationary phase in minimal media containing (i) 0.4% sodium gluconate and (ii) 0.4% sodium gluconate plus 0.4% glucose. The cells were harvested and washed three times with water. Portions corresponding to 50 mg (dry weight) were suspended in 1.0 ml of 1.0 N HCl; the tubes were closed with rubber stoppers and placed in a boiling-water bath for 3 hr. The debris was separated by centrifugation, the pellets were washed once with 1 ml of water, and the combined supernatant fluid and wash were neutralized with 10 N KOH. Glucose was assayed on portions of these hydrolysates. In an internal control, when 1 mg of glucose was added before hydrolysis to a sample of L40 grown on gluconate, 96% was recovered. Also, the quantities of hydrolysate used did not inhibit the glucose assay.

*Isolation and degradation of alanine.* Amounts of 0.01 ml from overnight cultures were inoculated in 25 ml of minimal medium containing 15.2 μmoles of glucose-*1*-$^{14}C$ or gluconate-*1*-$^{14}C$ per ml and incubated at 37 C on a shaker until fully grown. The cells (11 to 21 mg, dry weight, total) were harvested by centrifugation after acidification to 0.14 N trichloroacetic acid. Protein was prepared from them by the fractionation procedure of Roberts et al. (15). The protein fractions were hydrolyzed in 1.5 ml of 6 N HCl in sealed tubes at 109 C for 14 hr. The small amounts of debris were removed by centrifugation and washed with water; the washes and supernatant fluids were combined and taken to dryness several times in vacuo over solid KOH and concentrated $H_2SO_4$. Alanine was isolated from these hydrolysates by paper chromatography on Whatman no. 1 paper, descending, 19 hr by use of the *meta*-cresol-water system in an atmosphere of 1% $NH_3$ (3). Standards were located with a ninhydrin spray, and radioactivity in the samples was located with a Packard 4π chromatogram scanner. The band corresponding to alanine was eluted and chromatographed in a second system, *n*-butanol-acetic acid-water, 65:15:25 (18), Whatman no. 1 paper, descending, 12 hr. Alanine was eluted from the paper with 0.01 M acetic acid; its concentration was determined by the quantitative ninhydrin method of Troll and Cannon (18), and its radioactivity was determined in Bray's solution (2).

Radioactivity in the carboxyl group of alanine was determined by decarboxylation in the presence of carrier by the quantitative ninhydrin method done in a closed system, as follows. To a Thunberg tube 21 mm in diameter were added 0.1 ml (ca. 0.1 μmole) of radioactive sample, 0.05 ml of L-alanine-$^{12}C$ (10 μmoles/ml), 0.8 ml of the KCN-pyridine reagent (18), and 0.8 ml of 80% phenol (18). After chilling in ice, 0.2 ml of cold ninhydrin (50 mg/ml in ethyl alcohol) was quickly added, and the top, whose side arm contained 0.5 ml of 1 N KOH, was put on. The system was evacuated with a water pump and sealed when the liquid in the trap started to bubble (the reaction mixture being still yellow at this point). The reaction was started by putting the bottom 2.5 cm of the tube in a boiling-water bath. After 5 min, the tube was removed from the bath, and allowed to cool for a few minutes with occasional swirling to promote $CO_2$ trapping. The content of the trap, with two water washes, was transferred to a test tube, and the carbonate was precipitated by addition of 1 ml of saturated $Ba(OH)_2$. The precipitate was collected on B-6 membrane filters (Schleicher & Schuell Co., Keene, N.H.) and washed twice with 1 ml of water. The filters were dried for 15 min under a heat lamp, and their radioactivity was determined in a toluene scintillation system containing 2,5-diphenyloxazole (4 g per liter) and *p*-bis[2-(5-phenyloxazolyl)]-benzene (50 mg per liter). Radioactivity was measured with a Nuclear-Chicago Mark I scintillation counter equipped with an external standard which allowed all activities of samples counted in solution to be corrected to disintegrations per minute (dpm).

## RESULTS

*Selection of mutants lacking phosphoglucose isomerase.* Both the *S. typhimurium* (9) and the *E. coli* (10) mutants lacking phosphoglucose isomerase were first recognized as abnormal galactose-negative mutants. Our "rational" isolation of such mutants was based on the assumption that they would likely be negative or pseudo-negative on glucose indicator plates, and on minimal medium might show the growth pattern characteristic of the *Salmonella* mutant—slow growth on glucose (or galactose or maltose) and normal growth on carbon sources which are not metabolized via glucose-6-phosphate (fructose, gluconate, or glycerol).

*E. coli* strain K-10, $7 \times 10^7$ viable cells per ml in medium 63 was treated with 0.035 volumes of ethyl methane sulfonate (13) at 37 C for 20 min,

and dilutions were plated on glucose-tetrazolium plates. (On such plates, wildtype "fermentations" give pale colonies, whereas mutants are varying shades of red.) The viable count did not decrease significantly with mutagenesis, but the indicator plates from the mutagenized culture contained a small proportion of red colonies of various types. One hundred of these were purified and streaked on glucose, galactose, fructose, and gluconate minimal plates. Several classes of mutants could be recognized: some grew normally on glucose-minimal medium, others failed to grow on minimal medium at all, and yet others grew normally on some media but not on others. Among the latter were two, L40 and L93, which showed the same pattern previously seen with *Salmonella pgi* mutants. Enzyme assays on extracts prepared from cultures grown to stationary phase in broth showed that the parent strain had a phosphoglucose isomerase activity of 600 mμmoles per min per mg of protein, whereas L40 and L93 had activities of 2 and <1, respectively. Studies of revertants and mapping experiments with these strains show that they are both single gene mutations (7). In accord with the convention suggested by Demerec et al. (5) the mutant genes in these isolates will be called *pgi-2* and *pgi-3*. L40 (*pgi-2*) was chosen for further study.

*Growth studies.* Strains K-10 and L40 were inoculated on a variety of minimal media to give about 50 colonies per plate. As Table 1 shows, L40 formed substantially smaller colonies than K-10 on glucose, galactose, maltose, and lactose; on the other media, the colony sizes were more similar, though the mutant generally formed slightly smaller colonies than the parent strain.

Table 2 shows the growth rates of the two strains in liquid minimal media. The data are given from several determinations because the behavior of L40 on glucose was unusually variable. The cause of this variation is not known. In several experiments, L40 was subcultured repeatedly in glucose minimal medium, and the doubling times always increased, for example, from 144 min initially to 195 min after 16 generations. Further subcultures usually gave outgrowth of revertants. In a control with gluconate instead of glucose, the initial doubling time was 80 min, which after 24 generations was down to 64 min. Each single cycle of growth in such an experiment looked logarithmic. We conclude that L40 grows from one-half to one-third as fast as K-10 on glucose, slightly more slowly than K-10 on gluconate, and at an equal rate on fructose. The slow growth of the mutant on glucose is characterized by slow glucose utilization, since the efficiency of glucose utilization is similar in mutant and parent (Table 3).

*Glucose residue content.* Phosphoglucose isomerase has two roles: in growth on glucose, it must function in the direction of the conversion of glucose-6-phosphate to fructose-6-phosphate; in growth on gluconate or glycerol, on the other hand, it must function to convert fructose-6-phosphate to glucose-6-phosphate, which is a necessary step in the biosynthesis of several sugars

TABLE 1. *Colony size on several carbon sources*[a]

| Carbon source | Diam of colonies (mm) | |
|---|---|---|
| | Parent strain (K-10) | Mutant strain (L40) |
| Glucose | >2 | 0.5 |
| Galactose | 2.0 | 0.3 |
| Lactose | 1.8 | —[b] |
| Maltose | 1.5 | 0.3 |
| Acetate | 0.4 | 0.35 |
| Arabinose | 1.8 | 1.8 |
| Fructose | 1.4 | 1.3 |
| Gluconate | 2.0 | 1.3 |
| Glycerol | 1.2 | 0.9 |
| Succinate | 1.3 | 1.1 |

[a] Cultures were spread on minimal medium plates so as to give ca. 50 colonies per plate, and average colony size was estimated after 48 hr of incubation. All sugars, with the exception of (L) arabinose, were of the D configuration.

[b] Colonies on lactose appear with longer incubation.

TABLE 2. *Growth rates in minimal medium*[a]

| Doubling time (min) | | | | | |
|---|---|---|---|---|---|
| Glucose | | Fructose | | Gluconate | |
| Parent (K-10) | Mutant (L40) | Parent (K-10) | Mutant (L40) | Parent (K-10) | Mutant (L40) |
| 68 | 149 | 77 | 74 | 59 | 88 |
| 63 | 218 | 79 | 76 | 54 | 84 |
| 65 | 206 | | 95 | | |
| 63 | 121 | | | | |
| | 156 | | | | |
| | 150 | | | | |
| | 177 | | | | |
| Avg. 65 | 168 | 78 | 82 | 56 | 86 |

[a] The cultures were first grown to logarithmic phase in fructose minimal medium, and then were centrifuged and resuspended in minimal medium with the indicated carbon source. Each value represents a separate run. The rates were not different when the inocula were from stationary-phase cultures on fructose.

(e.g., glucose, galactose, and rhamnose) found in polysaccharides. Thus, if strain L40 is indeed deficient in phosphoglucose isomerase activity in vivo, in certain media this should be reflected in its polysaccharide content. Table 4 shows that, when the strains were grown on gluconate, the glucose found in an acid hydrolysate of washed cells in the mutant was about 2% of that in the parent, whereas after growth in the presence of glucose they had similar contents.

*Enzymes of glucose and gluconate metabolism.* Table 5 presents the activities of some enzymes of glucose metabolism in extracts prepared from cultures harvested from logarithmic growth on glucose, fructose, or gluconate. Phosphoglucose isomerase activity was constitutive in the parent strain, but it was absent in the mutant. The levels of glucokinase, glucose-6-phosphate dehydrogenase, and gluconate-6-phosphate dehydrogenase were similar in parent and mutant strain, and did not vary much with these growth conditions.

The mutant grew on gluconate only slightly more slowly than the parent strain (Table 2). Table 6 shows the levels of some enzymes of gluconate metabolism. Gluconokinase and gluconate-6-phosphate dehydrase were found, in both strains, only after growth on gluconate. KDGP aldolase, the second enzyme of the Entner-Doudoroff pathway, was found in high levels in all media, although there was some increase in the gluconate cultures. Thus, the metabolism of gluconate was effectively inducible by gluconate, since the kinase was inducible; and the metabolism of gluconate-6-phosphate by the Entner-Doudoroff path also would be expected to occur only in the presence of gluconate, since one of its enzymes was induced only in the presence of gluconate.

These results suggest that, in *E. coli*, glucose is metabolized via both phosphoglucose isomerase reaction and the hexose monophosphate shunt, whereas gluconate can be metabolized via the hexose monophosphate shunt and the Entner-Doudoroff pathway. In a *pgi* mutant, there was no evidence from these enzyme assays for derepression of either the hexose monophosphate shunt or the Entner-Doudoroff pathway, and the growth on glucose of such a mutant indeed appeared to be limited somehow by the capacity of the shunt.

TABLE 3. *Growth yields with glucose*[a]

| Expt | Cells formed (μg, dry wt) per mg of glucose used | |
|---|---|---|
| | Parent strain (K-10) | Mutant strain (L40) |
| 1 | 302 | 371 |
| 2 | 338 | 298 |
| 3 | 308 | 338 |
| Avg | 316 | 336 |

[a] The values were determined from plots of glucose in medium versus corrected turbidity (see Materials and Methods).

TABLE 4. *Content of glucose residues*[a]

| Medium | Glucose (μg/mg dry wt cells) | |
|---|---|---|
| | Parent (K-10) | Mutant (L40) |
| Gluconate | 13.1 | 0.2 |
| Gluconate and glucose | 44 | 37 |

[a] Glucose was determined in acid hydrolysates of cultures grown as indicated (see Materials and Methods).

*Tracer experiments.* The enzymatic data alone do not conclusively show that the Entner-Doudoroff pathway has no role in glucose metabolism, since it is conceivable that gluconate-6-phosphate dehydrase is present, but particularly unstable, in glucose-grown cells. We have therefore used specifically labeled radioactive substrates to independently estimate the use of several pathways in glucose and gluconate metabolism. The Entner-Doudoroff pathway was discovered in *Pseudomonas saccharophila* through experiments showing that gluconate labeled in the C-1 position could give rise to carboxyl-labeled pyruvate (6). Gluconate-*1*-$^{14}C$ used by the hexose monophosphate shunt ought to give unlabeled pyruvate, whereas glucose-*1*-$^{14}C$ used by glycolysis would give methyl-labeled pyruvate (see Fig. 1).

We have, therefore, done experiments which estimate the specific activity of pyruvate and the location of the radioactivity in the pyruvate molecule, during growth of strain K-10 on glucose-*1*-$^{14}C$ and on gluconate-*1*-$^{14}C$ and of strain L40 on glucose-*1*-$^{14}C$. To estimate the specific activities during growth, rather than in a nonphysiological situation, such as that of the arsenite-treated resting cells (6, 8), we have assumed that alanine comes from the transamination of pyruvate (19) and have isolated alanine from a protein hydrolysate prepared from cells grown for many generations on the various labeled carbon sources. The alanine was then degraded with ninhydrin to determine the proportion of radioactivity in the carboxyl group. The results are presented in Table 7. When K-10 was grown on glucose-*1*-$^{14}C$, the specific activity of the alanine was one-third that of the substrate, and a very small fraction of the counts in the alanine

were in the carboxyl group. When the same strain was grown on gluconate-*1*-$^{14}C$, the alanine had a specific activity about half that of the gluconate, and virtually all its counts were in the carboxyl group. When the mutant was grown on glucose-*1*-$^{14}C$, the alanine had a specific activity 8% that of the substrate; most of these counts were in the carboxyl group.

To interpret these data, it is assumed that pyruvate derived by different routes is sampled uniformly for alanine biosynthesis; i.e., there is no compartmentation. The interpretation is then as follows. Glucose is used by the wild-type strain largely by glycolysis, and partly by the hexose monophosphate shunt. Because only 3% of the counts in alanine are in the carboxyl group, the

TABLE 5. *Some enzymes of glucose metabolism*

| Enzyme | Carbon source | | | | | |
|---|---|---|---|---|---|---|
| | Glucose | | Fructose | | Gluconate | |
| | Parent (K-10) | Mutant (L40) | Parent (K-10) | Mutant (L40) | Parent (K-10) | Mutant (L40) |
| Glucokinase | 89 | 101 | 128 | 159 | 81 | 104 |
| Phosphoglucose isomerase | 1,070 | <1 | 1,280 | <1 | 954 | <1 |
| Glucose-6-phosphate dehydrogenase | 195 | 229 | 216 | 234 | 143 | 147 |
| Gluconate-6-phosphate dehydrogenase | 109 | 88 | 86 | 91 | 124 | 136 |

[a] Extracts were prepared from aerobic logarithmic phase cultures at 37 C. Enzyme activities are expressed as millimicromoles per minute per milligram of protein.

TABLE 6. *Some enzymes of gluconate metabolism*[a]

| Enzyme | Carbon source | | | | | |
|---|---|---|---|---|---|---|
| | Glucose | | Fructose | | Gluconate | |
| | Parent (K-10) | Mutant (L40) | Parent (K-10) | Mutant (L40) | Parent (K-10) | Mutant (L40) |
| Gluconokinase | 1 | 4 | 1 | 1 | 98 (213)[b] | 85 (211)[b] |
| Gluconate 6-phosphate dehydrase | 4 | 6 | 6 | 4 | 130 | 139 |
| KDGP aldolase | 208 | 96 | 131 | 86 | 370 | 465 |

[a] Extracts were prepared from aerobic logarithmic phase cultures at 37 C. Enzyme activities are expressed as millimicromoles per minute per milligram of protein.

[b] Values in parentheses were found when the assay system included 250 μg of protein of an extract from glucose-grown K-10 to supply excess gluconate-6-phosphate dehydrogenase (8).

TABLE 7. *Origin of alanine*[a]

| Strain | Carbon source | Specific activity of carbon source | Specific activity of alanine isolated | Per cent dpm in carboxyl group of alanine[b] |
|---|---|---|---|---|
| | | *dpm/μmole* | *dpm/μmole* | |
| Parent (K-10) | Glucose-*1*-$^{14}C$ | $4.33 \times 10^4$ | $1.47 \times 10^4$ | 3.6, 3.3 |
| Parent (K-10) | Gluconate-*1*-$^{14}C$ | $4.27 \times 10^4$ | $1.97 \times 10^4$ | 94, 97 |
| Mutant (L40) | Glucose-*1*-$^{14}C$ | $4.33 \times 10^4$ | $0.35 \times 10^4$ | 74, 78 |

[a] Alanine was isolated from cultures grown on the indicated carbon source; its specific activity and per cent radioactivity in the carboxyl group were determined (see Materials and Methods). When the decarboxylation was done on L-alanine-*U*-$^{14}C$ and *DL*-alanine-*1*-$^{14}C$, duplicate determinations gave 29.8 and 28.8% recovery of radioactivity from the former, and 89.0 and 96.8% from the latter.

[b] Duplicate determinations.

use of the Entner-Doudoroff path by the wild-type growing on glucose must be very small. The fraction using the shunt dilutes the specific activity of fructose-6-phosphate, and thus the specific activity of the pyruvate derived from glycolysis is lower than the 50% expected had all metabolism been via glycolysis.

When the wild-type grew on gluconate-*1*-$^{14}C$, its alanine was highly labeled in the carboxyl group, showing that a major portion of gluconate metabolism must be via the Entner-Doudoroff pathway. (It is not possible to estimate from these data the exact fraction using this pathway, because the extent of triose phosphate conversion to the pyruvate pool is not known.)

Finally, these data show that, when the mutant was grown on glucose-*1*-$^{14}C$, most of the glucose was metabolized by a pathway involving the loss of the 1-carbon atom: presumably, the hexose monophosphate shunt. However, since the counts in alanine were largely in the carboxyl group, a small fraction probably uses the Entner-Doudoroff path.

## DISCUSSION

The experiments reported in this paper have shown that *gluconate* metabolism in *E. coli* K-10 and in a phosphoglucose isomerase mutant derived from it is partly via the hexose monophosphate pathway and partly via the Entner-Doudoroff pathway. Earlier work with *S. typhimurium* (8) led to similar conclusions, which are also in accord with the report of Eisenberg and Dobrogosz on gluconate metabolism in *E. coli* ML30 (Bacteriol. Proc., 1966, p. 77).

*Glucose* metabolism in *E. coli* K-10 is via both the phosphoglucose isomerase reaction and the hexose monophosphate pathway. The *pgi*-negative mutant uses glucose chiefly by the hexose monophosphate pathway. The possible role of the Entner-Doudoroff pathway in *glucose* metabolism has been studied both by enzyme measurements and isotopic tracer experiments. There is no evidence for this pathway being used any more than very slightly by strain K-10 during growth on glucose. However, the mutant, after growth on glucose-*1*-$^{14}C$, contained a small but significant amount of radioactivity in the carboxyl group of alanine. According to results obtained with another mutant (20), no exchange reaction between radioactive $CO_2$ and unlabeled pyruvate would produce this much labeling in alanine, which must therefore be ascribed to minor use, by the mutant, of the Entner-Doudoroff pathway during growth on glucose. According to Table 6, the level of gluconate-6-phosphate dehydrase is very low in both parent and mutant, during logarithmic growth on glucose. It is not known whether this low level is sufficient to account for the minor use of the Entner-Doudoroff pathway by the mutant.

Indeed, the main discrepancy between our results and those of Loomis and Magasanik (12) concerns the inducibility of gluconate-6-phosphate dehydrase. They used stationary-phase cultures and found substantial activities in glucose-grown cells, whereas we used logarithmic-phase cultures and did not. Several results would be reconciled if it could be shown that gluconate-6-phosphate dehydrase appears only after logarithmic growth on glucose is over. However, in one experiment, we found no activity of this enzyme in a culture of our mutant in stationary phase on glucose. Thus, either it appears late and transiently, or there may be strain differences between derivatives of *E. coli* K-12.

The other differences between our results and Loomis and Magasanik's (12) concern the growth rates of phosphoglucose isomerase-negative mutants. These differences probably are not significant, for two reasons: (i) we found much variability in the growth rate of our mutant, and (ii) the mutant of Loomis and Magasanik, which we confirmed as *pgi*-negative, was derived in two steps (12) and may differ from the parent strain in more than one gene.

Since glucose was utilized with equal efficiency by our *pgi*-negative mutant and by its parent, the slow growth of the mutant reflected slow utilization of glucose, primarily via the hexose monophosphate pathway. It is not known why this pathway can only be used relatively slowly. Perhaps some enzyme is rate-limiting. Another possibility is that regeneration of nicotinamide adenine dinucleotide phosphate is the limiting step. We must also suggest a third factor possibly contributing to slow growth on glucose: the accumulation of some inhibitory metabolite. For, as might be expected from the properties of some other mutants blocked in catabolic pathways after the primary phosphorylation (e.g., references 1 and 4), in strain L40 glucose inhibits growth on fructose (but does not cause stasis). This problem is now being studied.

Further experiments on the role of the Entner-Doudoroff pathway in glucose and gluconate metabolism are presented in one accompanying paper (20), and genetic mapping of the phosphoglucose isomerase locus is presented in another (7).

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## LITERATURE CITED

1. BÖCK, A., AND F. C. NEIDHARDT. 1966. Properties of a mutant of *Escherichia coli* with a temperature-sensitive fructose-1,6-diphosphate aldolase. J. Bacteriol. **92**:470–476.
2. BRAY, G. A. 1960. A simple efficient liquid scintillator for counting aqueous solutions in a liquid scintillation counter. Anal. Biochem. **1**:279–285.
3. CONSDEN, R., A. H. GORDON, AND A. J. P. MARTIN. 1944. Qualitative analysis of proteins: a partition chromatographic method using paper. Biochem. J. **38**:224–232.
4. COZZARELLI, N. R., J. P. KOCH, S. HAYASHI, AND E. C. C. LIN. 1965. Growth stasis by accumulated L-α-glycerophosphate in *Escherichia coli*. J. Bacteriol. **90**:1325–1329.
5. DEMEREC, M., E. A. ADELBERG, A. J. CLARK, AND P. E. HARTMAN. 1966. A proposal for a uniform nomenclature in bacterial genetics. Genetics **54**:61–76.
6. ENTNER, N., AND N. DOUDOROFF. 1952. Glucose and gluconic acid oxidation of Pseudomonas saccharophila. J. Biol. Chem. **196**:853–862.
7. FRAENKEL, D. G. 1967. Genetic mapping of mutations affecting phosphoglucose isomerase and fructose diphosphatase in *Escherichia coli*. J. Bacteriol. **93**:1582–1587.
8. FRAENKEL, D. G., AND B. L. HORECKER. 1964. Pathways of D-glucose metabolism in *Salmonella typhimurium:* a study of a mutant lacking phosphoglucose isomerase. J. Biol. Chem. **239**: 2765–2771.
9. FRAENKEL, D., M. J. OSBORN, B. L. HORECKER, AND S. M. SMITH. 1963. Metabolism and cell wall structure of a mutant of *Salmonella typhimurium* deficient in phosphoglucose isomerase. Biochem. Biophys. Res. Commun. **11**:423–428.
10. HATTMAN, S. 1964. The functioning of T-even phages with unglucosylated DNA in restricting *Escherichia coli* host cells. Virology **24**:333–348.
11. LAYNE, E. 1957. Spectrophotometric and turbidimetric methods for measuring proteins, p. 454. *In* S. P. Colowick and N. O. Kaplan [ed.], Methods in enzymology, vol. 3. Academic Press, Inc., New York.
12. LOOMIS, W. F., JR., AND B. MAGASANIK. 1966. Nature of the effector of catabolite repression of β-galactosidase in *Escherichia coli*. J. Bacteriol. **92**:170-177.
13. LOVELESS, A., AND S. HOWARTH. 1959. Mutation of bacteria at high levels of survival by ethyl methane sulfonate. Nature **184**:1780–1782.
14. LOWRY, O. H., N. J. ROSEBROUGH, A. L. FARR, AND R. J. RANDALL. 1951. Protein measurement with the Folin phenol reagent. J. Biol. Chem. **193**:265–275.
15. ROBERTS, R. B., D. B. COWIE, P. H. ABELSON, E. T. BOLTON, AND R. J. BRITTEN. 1957. Studies of biosynthesis in *Escherichia coli*, p. 13–14. Carnegie Inst. Wash. Publ. 607.
16. SIGNER, E. R., J. R. BECKWITH, AND S. BRENNER. 1965. Mapping of suppressor loci in *Escherichia coli*. J. Mol. Biol. **14**:153–166.
17. SMITH, I. 1958. Chromatographic techniques, p. 19. Interscience Publishers, Inc , New York.
18. TROLL, W., AND R. K. CANNAN. 1953. A modified photometric ninhydrin method for the analysis of amino and imino acids. J. Biol. Chem. **200**: 803–811.
19. UMBARGER, E., AND B. D. DAVIS. 1962. Pathways of amino acid biosynthesis, p. 202–203. *In* I. C. Gunsalus and R. Y. Stanier [ed.], The bacteria, vol. 3, Biosynthesis. Academic Press, Inc., New York.
20. ZABLOTNY, R., AND D. G. FRAENKEL. 1967. Glucose and gluconate metabolism in a mutant of *Escherichia coli* lacking gluconate-6-phosphate dehydrase. J. Bacteriol. **93**:1579–1581.

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Auger EA, Bennett GN.

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*Short communication*

# Regulation of lysine decarboxylase activity in *Escherichia coli* K-12

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**Abstract.** The biodegradative lysine decarboxylase of *E. coli* has been reported to attain a higher specific activity when grown to saturation in the presence of excess lysine under conditions of low pH and absence of aeration. In order to examine possible sources of the pH and anaerobic regulation, a series of isogenic strains of *E. coli* K-12 were constructed. The effects of *cad*R$^-$, *fnr*$^-$, *cya*$^-$, *crp*$^-$ and *pgi*$^-$ mutations on lysine decarboxylase expression were examined. Cultures were grown in a lysine supplemented rich medium at pH 5.5, pH 6.8, and pH 8.0 with and without aeration and the enzyme was assayed from log phase cultures. The results suggested that the pH and air responses were independent and that these known regulatory processes are not responsible for this regulation of the biodegradative lysine decarboxylase.

**Key words:** Polyamines – pH regulation – Anaerobiosis – Cyclic AMP

In *Escherichia coli* two types of amino acid decarboxylases are found: the biosynthetic decarboxylases involved in the production of polyamines (Tabor and Tabor 1985) and the biodegradative or inducible decarboxylases acting on arginine, lysine and ornithine (Gale 1946). Studies designed to optimize enzyme production from high density cultures demonstrated that lysine decarboxylase levels were increased by the presence of the amino acid substrate, low pH, and absence of aeration (Gale 1946; Sabo et al. 1974). Gale (1946) discussed the possibility that these decarboxylases were important in maintaining the intracellular bicarbonate concentration under acidic conditions or in stabilizing the internal hydrogen ion levels. Recsei and Snell (1972) reported that the major defect of a histidine decarboxylase deficient strain of *Lactobacillus* 30A was the inability to regulate pH. Although genetic analysis revealed the inducible lysine decarboxylase is encoded by *cadA* (93 min) and is regulated by *cadR* in response to lysine (Tabor et al. 1980), the pH and anaerobic regulation of this enzyme has received little attention. Correlations of the expression of *cadA* and the nearby gene *lysU* have also been observed (Hershfield et al. 1984). One aspect of the mechanism of pH regulation of gene expression is its relation to other stress responses. Certain *lac* fusions have been isolated which showed a marked pH response (Aliabadi et al. 1986; Slonczewski et al. 1987). Induction of the SOS response (Schuldiner et al. 1986) or heat shock genes (Taglicht et al. 1987) by alkaline pH has been observed and the osmotic regulator *envZ* has been implicated in porin gene expression as a function of pH (Heyde and Portalier 1987).

## Materials and methods

### *Bacterial strains and media*

Bacterial strains used in this study are described in Table 1. CadR$^-$ strains were tested by plating on glucose minimal plates containing 25 μg/ml S-aminoethyl-L-cysteine (SAEC, Tabor et al. 1980). Fnr$^-$ strains were screened using the nitrate reductase overlay assay (Glaser and DeMoss 1972). ΔCya and ΔCrp strains were phenotypically scored by plating on MacConkey-lactose plus cAMP, lactose-minimal, and lactose-minimal plus cAMP. LB medium, Vogel-Bonner minimal medium, MacConkey indicator plates, and glucose tetrazolium plates were prepared as described (Miller 1972). The media were supplemented as necessary, in the following concentrations: cAMP 5 mM, methionine 40 mg/l, streptomycin 25 μg/ml, tetracycline 15 μg/ml, 2,3,5-triphenyl tetrazolium chloride 50 mg/l, and thiamine 2 mg/l.

### *Enzyme assays*

The strains were grown in a modified medium similar to the decarboxylase medium of Falkow (Falkow 1958) (0.5% Bacto-peptone, 0.5% L-lysine hydrochloride, 0.3% yeast extract, 1% D-glucose, and 50 mM buffer: MES for pH 5.5, MOPS for pH 6.8, and Tris for pH 8.0. For cultures grown under unaerated conditions, a 500 ml Erlenmeyer flask containing 500 ml of the modified Falkow medium was inoculated, sealed and grown at 37°C without agitation to an

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*Abbreviations: SAEC* S-aminoethyl-L-cysteine; *CRP* Cyclic AMP receptor protein

Table 1. Levels of lysine decarboxylase in HT177 derivatives

| Strain | Relevant Genotype | Specific activity of lysine decarboxylase[a] | | | | | |
|---|---|---|---|---|---|---|---|
| | | *Anaerobic growth* | | | *Aerobic growth* | | |
| | | pH 5.5 | pH 6.8 | pH 8.0 | pH 5.5 | pH6.8 | pH 8.0 |
| HT177 | cadR$^+$ fnr$^+$ | 150±53 (148± 1)[b] | 27±12 (21± 9)[b] | 1.3±0.7 (0.9±0.2)[b] | 8±4 | 5.0±0.9 | 0.06±0.03 |
| BAA17 | cadR$^+$ fnr$^-$ | 133±27 (138±30)[b] | 11± 5 (16± 4)[b] | 0.8±0.5 (1.0±0.1)[b] | 20±5 | 7.0±5.4 | 0.03±0.01 |
| HT316 | cadR$^-$ fnr$^+$ | 222±83 | 27±11 | 1.7±0.7 | 15±3 | 2.8±1.0 | 0.09±0.03 |

[a] Values in nmoles $^{14}CO_2$ evolved per minute per mg protein and are averages of five sets of assays
[b] Values in parenthesis are specific activities of lysine decarboxylase measured in unaerated cultures containing potassium nitrate (1 g/l)

$OD_{550}$ of 0.3 to 0.5. For cultures grown under aeration, a 1 l flask containing 250 ml of the modified Falkow medium was inoculated and grown at 37° C with agitation (275 rpm) in an air shaker incubator to an $OD_{550}$ of 0.3 to 0.5. During the growths the pH of the media changed less than 0.4 pH units toward pH 7. The cells were harvested, treated with 1% toluene, and prepared for lysine decarboxylase assays as described (Wertheimer and Leifer 1983). Lysine decarboxylase assays were performed as described (Tabor et al. 1980). Cell extracts were assayed for total protein using the Bradford protein assay from Bio-Rad (Bradford 1976).

## Results and discussion

A substantial increase in lysine decarboxylase specific activity was found in the absence of aeration and under low pH conditions even in early log phase cultures (Table 1). The low pH induction was observed in both the aerated and unaerated cultures. The data suggest that the pH effector can act with or without $O_2$ and does not require the presence of an active anaerobic regulator or the onset of stationary phase for its effect. These results also suggest that the pH and aeration regulatory processes are not due to pH or air regulated intracellular levels of lysine which then act directly through the lysine responsive *cadR* gene product. Although the *cadR* mutant used was reported to relieve lysine regulation of *cadA* (Tabor et al. 1980), it may not be completely devoid of the CadR protein, so the possibility that other mutant alleles or *cadR* deletions could yield a different result cannot be completely ruled out. Variations among strains exhibiting SAEC resistance have been described (Popkin and Maas 1980).

To determine if the aeration effect on level of the biodegradative lysine decarboxylase was mediated through the Fnr protein (Shaw and Guest 1982), a Fnr$^-$ derivative of HT177 was constructed by transduction of fnr-250 and an adjacent Tn*10* marker from RK5288 (J. DeMoss). There were no significant differences in lysine decarboxylase activity between the Fnr$^+$ and Fnr$^-$ strains in pH or aeration effects. Many anaerobically induced genes are repressed by anaerobically used electron acceptors, such as nitrate (Winkelman and Clark 1986). The addition of potassium nitrate to unaerated cultures did not alter the specific activity of lysine decarboxylase (Table 1).

In a similar experiment the effect of cyclic AMP and *pgi* (phosphoglucoseisomerase), a gene involved in anaerobic regulation in *S. typhimurium* (Jamieson and Higgins 1986), on regulation of lysine decarboxylase activity were studied using *Δcya*853, and *Δcrp*4 and *pgi*$^-$ derivatives of KC14, and F$^-$ *gal* strain from E. L. Kline. Under unaerated conditions the KC14, Δcya and Δcrp strains were similar in activity and lower activities were found in corresponding aerated cultures. The activity of the *pgi*$^-$ strain was lower than KC14 in aerated and unaerated cultures but the pH and aeration effects were still observed. Similar results were observed in modified Falkow medium (pH 5.5) containing fructose as the carbon source. These results suggest any effect of *pgi* was indirect.

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## References

Aliabadi Z, Warren F, Mya S, Foster JW (1986) Oxygen-regulated stimulons of *Salmonella typhimurium* identified by Mu d(Ap *lac*) operon fusions. J Bacteriol 165:780–786

Bradford M (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem 72:248–254

Falkow SN (1958) Activity of lysine decarboxylase as an aid in the identification of Salmonellae and Shigellae. Amer J Clin Path 29:598–600

Gale EF (1946) The bacterial amino acid decarboxylases. Adv in Enzymol 6:1–32

Glaser JH, DeMoss JA (1972) Comparison of nitrate reductase mutants of *Escherichia coli* selected by alternative procedures. Mol Gen Genet 116:1–10

Hershfield IN, Tenreiro R, Van Bogelen RA, Neidhardt FC (1984) *Escherichia coli* K-12 lysyl-tRNA synthase mutant with a novel reversion pattern. J Bacteriol 158:615–620

Heyde M, Portalier R (1987) Regulation of major outer membrane porin proteins of *Escherichia coli* K-12 by pH. Molec Gen Genet 208:511–517

Jamieson DJ, Higgins CF (1986) Two genetically distinct pathways for transcriptional regulation of anaerobic gene expression in *Salmonella typhimurium*. J Bacteriol 168:389–397

Lennox ES (1955) Transduction of linked genetic characters of the host by bacteriophage P1. Virology 1:190–206

Miller JH (1972) Experiments in molecular genetics. Cold Spring Harbor Laboratory, Cold Spring Harbor New York

Popkin PS, Maas WK (1980) *Escherichia coli* regulatory maturation affecting lysine transport and lysine decarboxylase. J Bacteriol 141:485–492

Recsei PA, Snell EE (1972) Histidine decarboxylaseless mutants of *Lactobacillus* 30a: Isolation and growth properties. J Bacteriol 112:624–626

Sabo DL, Boeker EA, Byers B, Waron H, Fischer EH (1974) Purification and physical properties of inducible *Escherichia coli* lysine decarboxylase. Biochemistry 13:662–670

Schuldiner S, Agmon V, Brandsma J, Cohen A, Friedman E, Padan E (1986) Induction of SOS functions by alkaline intracellular pH in *Escherichia coli*. J Bacteriol 168:936–939

Shaw DJ, Guest JR (1982) Nucleotide sequence of the *fnr* gene and primary structure of the Fnr protein of *Escherichia coli*. Nucl Acids Res 10:6119–6130

Slonczewski JL, Gonzalez TN, Bartholomew FM, Holt NJ (1987) Mu d-directed *lacZ* fusions regulated by acid pH in *Escherichia coli*. J Bacteriol 169:3001–3005

Tabor CW, Tabor H (1985) Polyamines in microorganisms. Microbiol Rev 49:81–99

Tabor H, Hafner EW, Tabor CW (1980) Construction of an *Escherichia coli* strain unable to synthesize putrescine, spermidine, or cadaverine: Characterization of two genes controlling lysine decarboxylase. J Bacteriol 144:952–956

Taglicht D, Padan E, Oppenheim AB, Schuldiner S (1987) An alkaline shift induces the heat shock response in *Escherichia coli*. J Bacteriol 169:885–887

Wertheimer SJ, Leifer Z (1983) Putrescine and spermidine sensitivity of lysine decarboxylase in *Escherichia coli*: Evidence for a constitutive enzyme and its mode of regulation. Biochem Biophys Res Commun 114:882–888

Winkelman JW, Clark DP (1986) Anaerobically induced genes of *Escherichia coli*. J Bacteriol 167:362–367

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